



In vitro antifungal effect of a plant-based product, CIN-102, on antifungal resistant filamentous fungi and their biofilms

Maurine d'Agostino, Nicolas Tesse, Rose Anne Lavergne, Patrice Le Pape, Jean Pol Frippiat, Marie Machouart, Anne Debourgogne

► To cite this version:

Maurine d'Agostino, Nicolas Tesse, Rose Anne Lavergne, Patrice Le Pape, Jean Pol Frippiat, et al.. In vitro antifungal effect of a plant-based product, CIN-102, on antifungal resistant filamentous fungi and their biofilms. *Journal of Medical Microbiology*, 2021, 70 (9), pp.768-773. 10.1099/jmm.0.001399 . hal-04482563

HAL Id: hal-04482563

<https://hal.univ-lorraine.fr/hal-04482563>

Submitted on 28 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

In vitro antifungal effect of a plant-based product, CIN-102, on antifungal resistant filamentous fungi and their biofilms

Maurine D'agostino^{1,*}, Nicolas Tesse², Rose Anne Lavergne^{3,4}, Patrice Le Pape^{3,4}, Jean Pol Frippiat¹, Marie Machouart^{1,5} and Anne Debourogne^{1,5}

Abstract

Introduction. The increase of invasive fungal infections (IFIs) and associated treatment failure in populations at risk is driving us to look for new treatments.

Hypothesis. The CIN-102 compound, derived from cinnamon essential oil, could be a new antifungal class with an activity, in particular, on strains resistant to current antifungals but also on biofilms, a factor of virulence and resistance of fungi.

Aim. The aim of this study is to show the activity of CIN-102 on various strains resistant to current antifungals, on the biofilm and to determine the possibility of resistance induced with this compound.

Methodology. We studied the MIC of CIN-102 and of current antifungals (voriconazole and amphotericin B) using CLSI techniques against eight different strains of three genera of filamentous fungi involved in IFIs and having resistance phenotypes to current antifungals. We also determined their effects on biofilm formation, and the induced resistance by voriconazole (VRC) and CIN-102.

Results. MIC values determined for CIN-102 were between 62.5 and 250 µg ml⁻¹. We demonstrated the antifungal effect of CIN-102 on biofilm, and more particularly on its formation, with 100% inhibition achieved for most of the strains. CIN-102 at a sub-inhibitory concentration in the medium did not induce resistance in our strains, even after 30 generations.

Conclusions. In this study we show that CIN-102 is effective against resistant filamentous fungi and against biofilm formation. In addition, our strains did not acquire a resistance phenotype against CIN-102 over time, unlike with VRC. CIN-102 is therefore an interesting candidate for the treatment of IFIs, including in cases of therapeutic failure linked to resistance, although further studies on its efficacy, safety and mechanism of action are needed.

INTRODUCTION

The incidence of invasive fungal infections (IFI) has been increasing over the past 20 years. Approximately 3600 people develop an IFI each year in France [1]. The emergence of these infections is due to an increase in antifungal prophylaxis or in immunosuppressive therapy, difficulties in clinical diagnosis, and the emergence of rare strains naturally resistant to the current antifungal arsenal [2]. However, only a few new antifungal drugs have been marketed in the last decade [3, 4].

Some fungi are almost entirely resistant to current antifungal drugs. For example, *Fusarium solani* and *Lomentospora prolificans* are considered minimally susceptible or non-susceptible to antifungals, respectively. Other fungi have acquired resistance, such as isolates belonging to the *Aspergillus fumigatus* complex, particularly to triazoles [5]. In these specific strains, the main genotypes observed are TR₃₄/L98H (substitution mutation of leucine 98 by a histidine in the CYP51A gene with a 34 bp tandem sequence in the promoter gene) and TR₄₆/Y121F/T289A (a 46 bp tandem repeat and Y121F and T289A

Received 15 March 2021; Accepted 05 June 2021; Published 07 September 2021

Author affiliations: ¹Université de Lorraine, SIMPA, F-54000 Nancy, France; ²Société Septeos, 12 avenue de la grande armée, 75017 Paris, France; ³Laboratoire de Parasitologie-Mycologie, Institut de Biologie, CHU de Nantes, France; ⁴Département de Parasitologie et Mycologie Médicale, Université de Nantes, Nantes Atlantique Universités, EA1155-ICiMed, Faculté de Pharmacie, Nantes, France; ⁵Université de Lorraine, CHRU-Nancy, laboratoire de Microbiologie, F-54000 Nancy, France.

*Correspondence: Maurine D'agostino, maurine.d-agostino8@univ-lorraine.fr

Keywords: Aspergillus; biofilm; Fusarium; Filamentous fungi; resistance; Scedosporium.

Abbreviations: AMB, Amphotericin B; BHIB, brain heart infusion broth; IFI, invasive fungal infection; LD50, lethal dose 50; MIC, minimum inhibitory concentration; MTR, multi triazole resistant; PBS, phosphate buffer saline; VRC, voriconazole; WT, wild-type.

001399 © 2021 The Authors



This is an open-access article distributed under the terms of the Creative Commons Attribution NonCommercial License.

Table 1. Summary of the fungal isolates studied, including origin, resistance phenotype, MIC CIN-102, voriconazole, amphotericin B and capacity to form biofilm

Strains	Origin	Resistance phenotype to antifungal	MIC determination ($\mu\text{g ml}^{-1}$)			Biofilm formation
			CIN-102	VRC	AMB	
<i>Aspergillus fumigatus</i>	ATCC 3626 California, United States	Sensitive	125	1	1	+
<i>Aspergillus fumigatus</i> TR34/L98h	Centre Hospitalier Universitaire Nantes	Resistant (Azoles)	250	8	0.5	+
<i>Aspergillus fumigatus</i> TR46 Y121F T289A	Centre Hospitalier Universitaire Nantes	Resistant (Azoles)	250	16	0.5	+
<i>Aspergillus flavus</i>	ATCC 204304 Human sputum, Virginia	Sensitive	125	0.5	1	+
<i>Fusarium solani</i>	CBS 124631 Ongle France, Paris	Resistant (Azoles)	62.5	4	2	++
<i>Fusarium dimerum</i>	NCPF 7449 Human, blood culture	Resistant (Azoles)	62.5	8	1	++
<i>Scedosporium apiospermum</i>	ATCC 3635 Human	Sensitive	62.5	0.5	1	+
<i>Lomentospora prolificans</i>	Centre Hospitalier Régional Universitaire de Nancy	Resistant (Azoles, Amphotericin B)	62.5	8	8	++

AMB, amphotericin B; IFI, invasive fungal infection; MTR, multi triazol resistant; VRC, Voriconazole.

substitutions) [6]. Such resistance emerges in the environment or in patients themselves during antifungal treatment.

The formation of a biofilm can also contribute to antifungal resistance. A biofilm is an irreversible association of microbial cells, constituting a surface embedded in an extracellular matrix. Biofilm-forming organisms differ from their planktonic forms in terms of gene expression or shape. Biofilms can form over a large number of surfaces, whether organic or not, such as living tissue, medical devices, tubing, etc [7]. Pathogens embedded in biofilms are known to be much more resistant to antimicrobial agents than their planktonic counterparts. Many factors are involved in this phenomenon, such as reduced diffusion due to the exopolysaccharide matrix or increased cell density. In addition, biofilm formation induces changes in gene expression, including modifications in the expression of antifungal resistance genes [8]. The presence of persistent (dormant) cells resistant to antifungal treatment also plays a role in the overall resistance of biofilms to antimicrobials [9].

Thus, many situations can interfere with the drugs currently available in clinical practice, and new classes of antifungals are needed. The resources of the plant world in active substances used for therapeutic purposes are significant, although still underexploited. Essential oils extracted from plants (leaves, flowers, roots or barks) have different pharmacological properties such as anti-infectious, antioxidant effects and antifungal properties [10, 11]. Recent studies have produced promising data concerning the effect of

cinnamaldehyde (a major compound of cinnamon essential oil) on the main fungal genera involved in human pathology, or on toxigenic fungi [12–15]. The Septeos company has developed CIN-102, a synthetic mixture of seven compounds comprising three cinnamaldehyde derivatives, that was developed from the formulation of cinnamon essential oil available in the Pharmacopoeia, and from which the genotoxic compounds (safrole, eugenol and coumarin) have been removed. The compounds in this mixture are synergic, and exhibit fungicidal antifungal activity on the genera *Aspergillus*, *Fusarium* and *Scedosporium* [16]. Consequently, this study aimed to determine the effect of this new compound on the resistance phenotypes of strains involved in IFIs, on biofilm formation, and on induced resistance.

METHODS

Fungal isolates

Strains of filamentous fungi of *Aspergillus* ($n=4$), *Fusarium* ($n=2$) and *Scedosporium* ($n=2$) genera from different origins (clinical and reference strains) and resistance phenotypes to voriconazole were used in this study. Both isolates of multi triazole resistant (MTR) *Aspergillus* strains were tested: *Aspergillus fumigatus* TR₄₆/Y121F/T289A and *Aspergillus fumigatus* TR₃₄/L98H (denoted TR₄₆ and TR₃₄ respectively). The fungal isolates, their origin, their MIC to CIN-102, voriconazole (VRC), and amphotericin B (AMB), as well as their capacity to form biofilm are presented in Table 1.

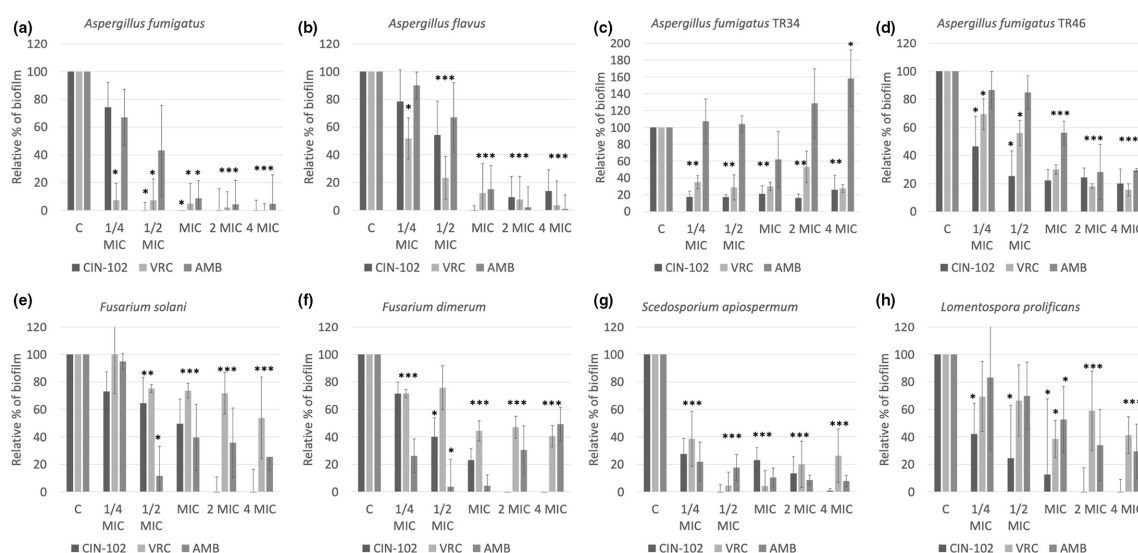


Fig. 1. (a–h) Relative percentage of biofilm formation measured by XTT assay for the different isolates studied after treatment with CIN-102, voriconazole or amphotericin b. * Results significantly different (P -value < 0.05) from the control (denoted c).

Culture and storage

Strains were subcultured on Sabouraud agar medium (Merck Sigma Aldrich, Darmstadt, Germany). After 48 to 72 h of incubation at 31 °C, colonies were picked using sterile swabs from the medium and suspended in 0.9% NaCl or phosphate buffered saline (PBS) (for biofilm studies) solutions. Strains were stored and preserved in a mixture containing 1 ml of glycerol (Merck Sigma Aldrich, Darmstadt, Germany) and 1 ml of 0.9% NaCl at –20 °C.

MIC determination

The CLSI M38-A2 reference protocol was followed [17]. Two antifungal agents included in the protocol, amphotericin B and voriconazole (Merck Sigma Aldrich, Darmstadt, Germany) (dilution range 0.03 to 16 µg ml⁻¹) were used in addition to CIN-102 (Septeos, Paris, France). After a preliminary test, the dilution range chosen for CIN-102 extended from 1.9 to 1000 µg ml⁻¹. After culture, conidia were suspended in 0.9% NaCl filtered, adjusted to 5 × 10⁴ conidia ml⁻¹ using a spectrometer at 530 nm, and diluted to 1:50 to obtain the working suspensions.

An inoculum of 100 µl was distributed in 96-well plates containing antifungal dilutions. These plates were incubated at 37 °C for 48 h. All strains were tested in duplicate. The MIC was determined as the lowest drug concentration allowing complete growth inhibition after 24 to 48 h of incubation. Each 96-well plate contained a control strain (*Aspergillus fumigatus* ATCC 3626, *Aspergillus flavus* ATCC 204304 or *Scedosporium apiospermum* ATCC 3635), as indicated in the CLSI protocol. A plastic film was placed on every plate to limit evaporation and contamination. The density of the inoculum was checked by seeding 10 µl of the working suspension diluted to 1:10 on Sabouraud agar medium after a 1 day incubation at 31 °C. One to ten colonies had to be counted to validate the test.

Differences between MICs were considered significant when a difference greater than two ranks of dilution was observed.

Biofilm formation and semi-quantification

Crystal violet staining was used to visualize the formation of biofilm using the protocol of Sav *et al.* [18]. A suspension of conidia was made for each strain and adjusted to 10⁴ cells ml⁻¹ in PBS. One hundred microliters of this suspension were added to 2 ml of brain heart infusion broth (BHIB) with glucose (0.25%). After 24 h of incubation at 37 °C, the suspension was diluted 1:20 in fresh BHIB. Two hundred microliters of this suspension were placed in a 96-well plate which was incubated for 24 h at 37 °C. After incubation, the wells were washed three times with PBS and 200 µl of 1% crystal violet was added. After 15 min of incubation at room temperature, the wells were rinsed three times with PBS and 200 µl of ethanol 100% was added. The absorbance was measured at 450 nm by a plate reader (SpectreMax iD3, Molecular Device, San José, United States). The percentage of transmittance (% T) of each test sample was subtracted from the % T of the reagent blank to obtain a measure of the relative amount of light blocked by the sample (% T_{block}). A semi-quantitative evaluation of biofilm formation was carried out using the following values of % T_{block}: negative: % T_{block} < 5, + (positive): 5 < % T_{block} < 20, ++: 20 < % T_{block} < 50, +++: % T_{block} > 50. The biofilm activity of *C. albicans* ATCC 90028 (5 < % T_{block} < 20) was used as a positive quality control.

Antifungal effect on biofilm formation

The effect of antifungal compounds on biofilm formation was measured using XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide). This assay is based on the reduction of the tetrazolium salt

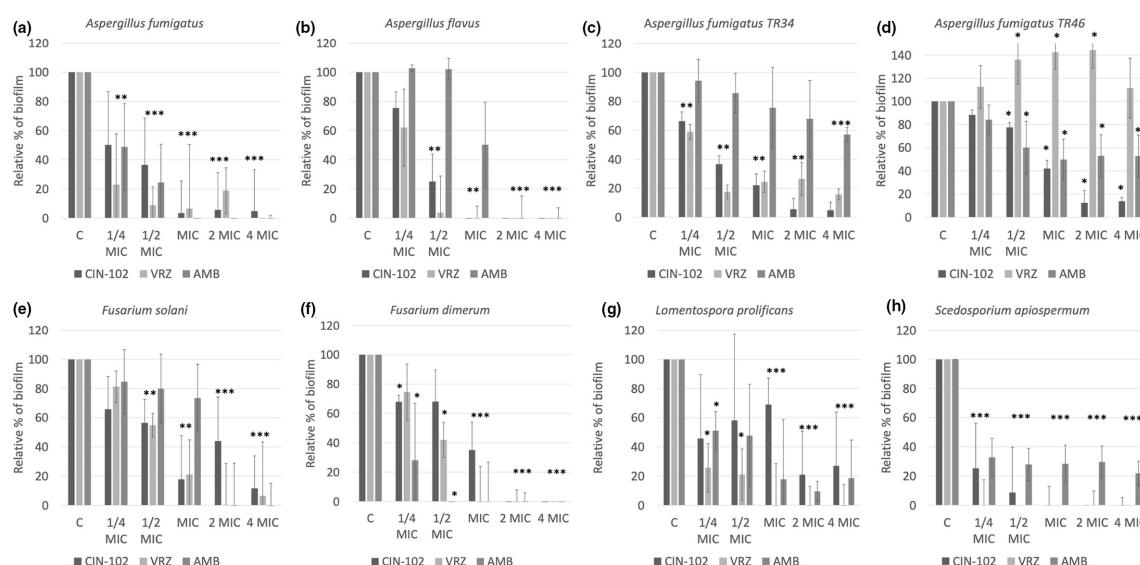


Fig. 2. (a–h): Relative percentage of preformed biofilm measured by XTT assay for the different strains studied after treatment with CIN-102, voriconazole or amphotericin b. Different concentrations of antifungals (1/4 MIC, 1/2 MIC, MIC, 2 MIC and 4 MIC) are added to the medium after twenty-four hours of biofilm growth. * Results significantly different (P -value < 0.05) from the control (denoted c).

XTT to a water soluble orange-coloured formazan compound by metabolically active cells [19].

In order to induce cell adhesion, 100 μ l of a suspension containing 10^4 cells ml^{-1} in PBS were placed in the wells of a 96-well plate at 37°C for 90 min. Then, in each well, 100 μ l of RPMI 1640 with MOPS medium (US Biological, United States) or an antifungal quantity (CIN-102, AMB or VRC) corresponding to 1/4, 1/2, 1, 2 and 4 MIC were added. A well without antifungals served as positive control. This plate was incubated at 37°C for 24 h. A mixture of one hundred microliters of XTT (Merck Sigma Aldrich, Darmstadt, Germany) and menadione (Merck Sigma Aldrich, Darmstadt, Germany) was prepared to achieve concentrations of 0.5 g l^{-1} and 1 mM, respectively, and added to each well. After 1 h of incubation at 37°C, the optical density was measured (SpectraMax iD3, Molecular Device) at 450 and 630 nm. For each well, the conversion of XTT was determined by subtracting the blank OD (mixture of XTT and menadione in a well without biofilm) and the non-specific absorbance measured at 630 nm from the sample OD. The percentage of biofilm formation was calculated by determining the ratio between XTT conversion in each well and XTT conversion in the drug-free control well.

Antifungal effect on preformed biofilms

After biofilm formation [18], steam washing (40 min) [20] and drying (30 min), 200 μ l of RPMI was added to the wells, with different concentrations of antifungals (amphotericin B, voriconazole, CIN-102 at 1/2, 1 and 4 MIC). Each concentration was tested in triplicate, and three wells per strain without antifungals served as positive controls. After 24 h of incubation at 37°C, 100 μ l of the XTT +menadione mixture described above were added to each well containing the pre-washed biofilms. After reading the OD at 450 and 630 nm, the

same calculation as above was carried out to determine the percentage of biofilm formation relative to controls. All wells were prepared in duplicate and the experiment was repeated three times.

Induced resistance

To determine whether the presence of a sub-inhibitory concentration of antifungal in the medium induces resistance, cultures were seeded over three generations in the presence of antifungals, with a determination of the MIC for each generation. The different strains were cultured in 40 ml of Sabouraud dextrose broth (SBD) medium [21]. An antifungal (VRC or CIN-102) was added to the culture at a concentration equal to 0, 1/4 or 1/2 of the initial MIC of the strain. After 3 days of incubation at 37°C with shaking, part of the culture was resuspended in fresh medium containing the same concentration of antifungals and was again incubated at 37°C for 3 days with shaking, thus forming the second generation. The other part of the culture was homogenized by sonication (Vibra-cell, Sonics and Materials, Inc., Newtown, United States) and then diluted 1:10 before MIC determination. These analyses were performed in triplicate and the experiment was performed three times.

To validate the obtained results and to study the induction of resistance over numerous generations, the strains were inoculated on solid SBD medium containing 1/2 MIC of CIN-102 and were incubated at 37°C. The strains were sub-cultured onto a new plate every 3 days. After ten generations, the culture was suspended in 0.9% NaCl, adjusted to 10^4 cells ml^{-1} , and determination of the MIC was carried out.

Table 2. MIC determined for CIN-102 and voriconazole, following three generations in the presence of a subinhibitory concentration of CIN-102 or voriconazole

		Voriconazole MIC in (μg ml ⁻¹)		CIN-102 MIC in (μg ml ⁻¹)		
<i>A. fumigatus</i>	Control	1/2 MIC	1/4 MIC	Control	1/2 MIC	1/4 MIC
Generation 1	0.5	0.5	0.5	125	125	125
Generation 2	0.5	2	2	125	62.5	62.5
Generation 3	0.5	8	8	125	62.5	62.5
<i>A. flavus</i>	Control	1/2 MIC	1/4 MIC	Control	1/2 MIC	1/4 MIC
Generation 1	1	1	1	500	500	500
Generation 2	1	8	8	250	250	125
Generation 3	2	8	8	250	250	250
<i>F.solani</i>	Control	1/2 MIC	1/4 MIC	Control	1/2 MIC	1/4 MIC
Generation 1	4	4	4	250	250	250
Generation 2	4	16	16	250	250	250
Generation 3	4	16	16	250	250	250
<i>F. dimerum</i>	Control	1/2 MIC	1/4 MIC	Control	1/2 MIC	1/4 MIC
Generation 1	8	8	8	250	125	125
Generation 2	8	16	32	125	125	125
Generation 3	8	16	32	250	250	125
<i>S. apiospermum</i>	Control	1/2 MIC	1/4 MIC	Control	1/2 MIC	1/4 MIC
Generation 1	1	1	1	125	125	125
Generation 2	0.5	4	4	62.5	62.5	62.5
Generation 3	0.5	8	8	62.5	62.5	62.5
<i>L. prolificans</i>	Control	1/2 MIC	1/4 MIC	Control	1/2 MIC	1/4 MIC
Generation 1	16	16	16	62.5	62.5	62.5
Generation 2	16	32	16	62.5	62.5	62.5
Generation 3	16	48	48	62.5	62.5	62.5

BHIB, brain heart Infusion broth; MIC, minimal inhibitory concentration; PBS, phosphate buffer saline.

Statistical analysis

Data analysis was performed using XLSTAT (XLSTAT statistical and data analysis solution, New York, USA. <https://www.xlstat.com>). Differences between groups were determined using Student's *t* tests and considered statistically significant at *P* values ≤ 0.05 .

RESULTS

MIC determination

CIN-102 and AMB MIC values were $250 \mu\text{g ml}^{-1}$ and $0.5 \mu\text{g ml}^{-1}$, respectively, for these strains (Table 1). No significant difference between susceptible (MIC $125 \mu\text{g ml}^{-1}$ and $1 \mu\text{g ml}^{-1}$) or resistant strains were noted for CIN-102

and AMB. However, there was a significant difference between MTR and wild-type (WT) strains of *Aspergillus fumigatus* to VRC. The average MIC for susceptible strains was $1 \mu\text{g ml}^{-1}$, while it was 8 or $16 \mu\text{g ml}^{-1}$ for *A. fumigatus* TR₃₄ and TR₄₆, respectively, indicating a dilution rank greater ≥ 2 [16].

The genera *Fusarium* and *Scedosporium* are considered as not very susceptible to antifungals. The average MIC for CIN-102 was $62.5 \mu\text{g ml}^{-1}$ for these strains. In contrast, a significant difference was found between species of *Scedosporium* and *Fusarium* genera regarding VRC and AMB, with MIC values ranging from 0.5 to $8 \mu\text{g ml}^{-1}$ and 0.5 to $2 \mu\text{g ml}^{-1}$ for VRC and AMB, respectively.

Table 3. MIC determined for fungal isolates against CIN-102, following 30 generations on solid culture medium in the presence of a sub-inhibitory concentration of CIN-102

Strains	After 10 generations		After 20 generations		After 30 generations	
	Control ($\mu\text{g ml}^{-1}$)	Test ($\mu\text{g ml}^{-1}$)	Control ($\mu\text{g ml}^{-1}$)	Test ($\mu\text{g ml}^{-1}$)	Control ($\mu\text{g ml}^{-1}$)	Test ($\mu\text{g ml}^{-1}$)
<i>A. fumigatus</i>	250	250	125	250	250	250
<i>A. flavus</i>	250	250	125	125	250	250
<i>F. solani</i>	125	125	125	125	125	250
<i>F. dimerum</i>	125	125	125	125	125	125
<i>L. prolificans</i>	125	125	125	125	125	125
<i>S. apiospermum</i>	125	125	125	125	125	125

Biofilm formation

Before evaluating the activity of CIN-102 on biofilm, we checked that the strains used in this study were capable of producing a biofilm (Table 1). All our strains formed biofilms in moderate quantities. *F. solani*, *F. dimerum* and *L. prolificans* had a %Tbloc slightly above 20%, while *A. fumigatus*, *A. fumigatus* TR₃₄ and TR₄₆, *A. flavus* and *S. apiospermum* had a %Tbloc between 15 and 20%.

Effect of antifungals on biofilm formation

The effect of CIN-102 on biofilm formation was studied and compared with those of VRC and AMB (Fig. 1). We noticed a dose-dependent decrease for most of the strains (for *A. fumigatus*, *A. flavus*, *F. solani*, *F. dimerum* and *L. prolificans* with CIN-102, for WT and MTR *Aspergillus fumigatus* strains, *A. flavus*, *F. solani* and *L. prolificans* with VRC and for *A. flavus*, *A. fumigatus* TR₄₆, *F. solani* and *L. prolificans* with AMB). In addition, VRC and AMB appeared to be less effective in inhibiting biofilm formation than CIN-102. On *Aspergillus* TR₃₄ and TR₄₆ MTR strains, VRC inhibited only 70 and 80% of biofilm formation and AMB achieved at best 60 and 70% of biofilm inhibition, respectively. In comparison, CIN-102 inhibited at least 90% of biofilm formation by these two strains. Finally, CIN-102 was able to inhibit 100% of biofilm formation by five other tested strains (*A. fumigatus*, *A. flavus*, *F. dimerum*, *L. prolificans* and *S. apiospermum*) against one for VRC (*A. fumigatus*) and two for AMB (*A. fumigatus* and *A. flavus*) and it was effective at low concentration for all of the strains (from 1/2 MIC to MIC).

Effect of antifungals on preformed biofilms

Elimination of preformed biofilms was evaluated after contact with CIN-102 and other antifungal agents (Fig. 2). A dose-dependent decrease was observed with CIN-102 for all strains except *L. prolificans*, where the decrease occurred around 1 MIC. A one hundred percent elimination was reached for *Fusarium dimerum*, *A. flavus* and *S. apiospermum* at 1 or 2 MIC, more than 90% of reduction was reached for WT or MTR *Aspergillus fumigatus* strains, and 80% for *L. prolificans* and *F. solani* at the same MIC values. A dose-dependent decrease was also observed with VRC for almost all the strains except for *A. fumigatus* TR₄₆. One hundred percent of reduction was

reached at 1/2 MIC for *S. apiospermum*, at 1 MIC for *A. flavus*, *F. dimerum* and *L. prolificans*, at 2 MIC for *F. solani* and at 4 MIC for *A. fumigatus*. Finally, all strains treated with AMB had a dose-dependent decrease of preformed biofilm. A one hundred percent elimination of preformed biofilm was achieved for *F. dimerum* and *S. apiospermum* at 1/2 MIC, and for *A. fumigatus* and *A. flavus* at 1 and 2 MIC, respectively. It is important to note that CIN-102 inhibited at least 90% of preformed biofilm of *Aspergillus fumigatus* MTR strains and was as effective as the other antifungals for the other strains.

Induced resistance

Induced resistance was observed in all strains tested with VRC (Table 2). Between first and third generation, MIC evolved from 0.5 $\mu\text{g ml}^{-1}$ to 8 $\mu\text{g ml}^{-1}$ in the presence of 1/2 and 1/4 MIC of VRC for *Aspergillus fumigatus*; from an MIC of 1 to 8 $\mu\text{g ml}^{-1}$ for *Aspergillus flavus*; from 8 to 32 $\mu\text{g ml}^{-1}$ for *Fusarium dimerum*; from 4 to 16 $\mu\text{g ml}^{-1}$ for *Fusarium solani*; from 1 to 8 $\mu\text{g ml}^{-1}$ for *S. apiospermum*; and finally, from 16 to 32 or 48 $\mu\text{g ml}^{-1}$ for *L. prolificans*. In contrast, for CIN-102, there was no significant difference between the different generations. In addition, there was no induced resistance after 30 generations in the presence of CIN-102 for all tested strains (Table 3).

DISCUSSION

In a previous study we demonstrated that the distribution of MIC for CIN-102 was unimodal for *Fusarium*, *Scedosporium* and *Aspergillus* genera, with a small range of MIC values, unlike voriconazole and amphotericin B (3–4 dilution range of MIC for CIN-102). These results indicate that these strains did not develop resistance against CIN-102 [16]. We also demonstrated that CIN-102 is equally effective against both susceptible and resistant strains, unlike VRC and AMB. The increased resistance of biofilms to antifungals has been previously shown [22], especially for *Candida* and *Aspergillus* strains. For example, Muckherjee *et al.* demonstrated an MIC greater than 256 $\mu\text{g ml}^{-1}$ for the biofilm form compared to 0.5 $\mu\text{g ml}^{-1}$ for the planktonic form of *Candida albicans* with VRC [23]. For *Aspergillus fumigatus*, the MIC ranged from 0.25 to 1 $\mu\text{g ml}^{-1}$ for the planktonic phase to 16 to 128 $\mu\text{g ml}^{-1}$

for the biofilm with the same antifungal [24]. On the other hand, it seems that biofilm resistance is less significant with AMB [23–25], as confirmed by our experiments performed with *F. solani*, *L. prolificans* and *Aspergillus* strains. Our study also demonstrated that VRC was less effective for inhibiting *Fusarium* and *L. prolificans* biofilm formation, and for inhibiting *Aspergillus* and *S. apiospermum* preformed biofilms. This last observation is in line with the literature, since VRC is described as being more effective against dividing cells [24]. CIN-102, on the other hand, appears to be more effective against developing biofilms, and is generally more effective at removing biofilm than VRC and AMB.

It has already been demonstrated several times that azole antifungals can induce resistance in treated strains. This induced resistance can be rapid (after 2 days of contact) [26] and can take place even in the presence of a low concentration of the antifungal in the medium (below the MIC) [21, 27]. Induced resistance can be stable over time (greater than 30 days) [26] and can cross between different azole antifungals (for example, a triazole antifungal used in agriculture can induce cross resistance to clinical triazoles) [21, 28]. Our results confirmed this rapid induction of resistance at low concentrations for voriconazole. On the other hand, no resistance was detected with CIN-102, even after 30 days of contact. In general, very few cases of resistance have been described for essential oils [29–31].

Individual component toxicities of the components of CIN-102 are well known (the oral LD 50 determined for rats is 2.2 g kg⁻¹ according Fisher Scientific, safety data) and seems compatible with human use from an acute toxicity point of view. The Septeos company has already established the absence of genotoxicity *in vitro* and *in vivo*, confirming potential use in humans. Nevertheless, precise monitoring of toxicity in an animal model is required.

CONCLUSION

This study reinforces the potential of CIN-102 as a new antifungal because *i*) we were able to demonstrate its effects on strains considered less sensitive to conventional antifungal agents, *ii*) no resistance or induced resistance was observed, unlike other antifungals tested, and *iii*) cinnamaldehyde is known to have low toxicity, suggesting that CIN-102 will itself be of low toxicity. These results are promising to offset the problem of treatment failure due to resistance to current first-line antifungals in the treatment of IFIs.

Funding information

This work was supported by a CIFRE grant with SEPTEOS.

Acknowledgements

We would like to thank the French Ministry of Higher Education and Research, the Université de Lorraine, and the CPER IT2MP (Contrat Plan État Région, Innovations Technologiques, Modélisation and Médecine Personnalisée) and FEDER (Fonds européen de développement régional). The authors acknowledge support of the Stress, Immunity, Pathogens unit by the 'Impact Biomolecules' project of the 'Lorraine Université d'Excellence' (Investissements d'avenir – ANR).

Author contributions

Conceptualisation: M. D., A. D. and N. T., Methodology: M. D., A. D., Validation: M. D., A. D., Formal Analysis: M. D., Investigation: M. D., Resources: J. P. F., N. T., P. P. and R. A. L., Writing – Original Draft Preparation: M. D., Writing – Review and Editing: M. D., A. D., N. T. and J. P. F., Supervision: A. D., Funding: N. T.

Conflicts of interest

This work was funded by a grant received from the Septeos Company.

References

1. Bitar D, Lortholary O, Le Strat Y, Nicolau J, Coignard B, et al. Population-based analysis of invasive fungal infections, France, 2001–2010. *Emerg Infect Dis* 2014;20:1149–1155.
2. Blot M, Lanternier F, Lortholary O. Epidemiology of visceral fungal infection in France and in the world. *Rev Prat* 2015;65:1318–1321.
3. Rapp RP. Changing strategies for the management of invasive fungal infections. *Pharmacotherapy* 2004;24:4S–28S.
4. Robbins N, Caplan T, Cowen LE. Molecular evolution of antifungal drug resistance. *Annu Rev Microbiol* 2017;71:753–775.
5. Lee HJ, Cho SY, Lee D-G, Park C, Chun HS, et al. TR34/L98H mutation in cyp51a gene in *Aspergillus fumigatus* clinical isolates during posaconazole prophylaxis: First case in Korea. *Mycopathologia* 2018;183:731–736.
6. van der Linden JWM, Camps SMT, Kampinga GA, Arends JPA, Debets-Ossenkopp YJ, et al. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin Infect Dis* 2013;57:513–520.
7. Donlan RM. Biofilms: microbial life on surfaces. *Emerging Infect Dis* 2002;8:881–890.
8. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, et al. Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. *J Bacteriol* 2001;183:5385–5394.
9. LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob Agents Chemother* 2006;50:3839–3846.
10. D'agostino M, Tesse N, Frippiat JP, Machouart M, Debourgogne A. Essential oils and their natural active compounds presenting antifungal properties. *Molecules* 2019;24.
11. Mirza Alizadeh A, Golzan SA, Mahdavi A, Dakhili S, Torki Z, et al. Recent advances on the efficacy of essential oils on mycotoxin secretion and their mode of action. *Crit Rev Food Sci Nutr* 2021;1–26.
12. Shreaz S, Wani WA, Behbehani JM, Raja V, Irshad M, et al. Cinnamaldehyde and its derivatives, a novel class of antifungal agents. *Fitoterapia* 2016;112:116–131.
13. Liang D, Xing F, Selvaraj JN, Liu X, Wang L, et al. Inhibitory effect of cinnamaldehyde, citral, and eugenol on aflatoxin biosynthetic gene expression and aflatoxin B1 biosynthesis in *Aspergillus flavus*. *J Food Sci* 2015;80:M2917–24.
14. Morcia C, Tumino G, Ghizzoni R, Bara A, Sathi N, et al. *In vitro* evaluation of sub-lethal concentrations of plant-derived antifungal compounds on fusaria growth and mycotoxin production. *Molecules* 2017;22.
15. Khan MSA, Ahmad I. Antifungal activity of essential oils and their synergy with fluconazole against drug-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*. *Appl Microbiol Biotechnol* 2011;90:1083–1094.
16. D'agostino M, Tesse N, Lavergne RA, Pape PL, Bouchara JP, et al. Characterization of the antifungal effect of a plant-based compound, CIN-102, on the main septal filamentous fungi involved in human pathology. *J Glob Antimicrob Resist* 2021;25:171–180.
17. Wayne P. *CLSI Document M38-A2*. 2008.
18. Sav H, Rafati H, Öz Y, Dalyan-Cilo B, Ener B, et al. Biofilm formation and resistance to fungicides in clinically relevant members of the fungal genus *Fusarium*. *J Fungi (Basel)* 2018;4.

19. Kuhn DM, Balkis M, Chandra J, Mukherjee PK, Ghannoum MA. Uses and limitations of the XTT assay in studies of Candida growth and metabolism. *J Clin Microbiol* 2003;41:506–508.
20. Tasse J, Cara A, Saglio M, Villet R, Laurent F. A steam-based method to investigate biofilm. *Sci Rep* 2018;8:13040.
21. D'Agostino M, Lemmet T, Dufay C, Luc A, Fripiat JP, et al. Over-induction of CYP51A gene after exposure to azole antifungals provides a first clue to resistance mechanism in *Fusarium solani* species complex. *Microb Drug Resist* 2018;24:768–773.
22. Kowalski CH, Morelli KA, Schultz D, Nadell CD, Cramer RA. Fungal biofilm architecture produces hypoxic microenvironments that drive antifungal resistance. *Proc Natl Acad Sci USA* 2020;117:22473–22483.
23. Mukherjee PK, Chandra J. Candida biofilm resistance. *Drug Resist Updat* 2004;7:301–309.
24. Mowat E, Lang S, Williams C, McCulloch E, Jones B, et al. Phase-dependent antifungal activity against *Aspergillus fumigatus* developing multicellular filamentous biofilms. *J Antimicrob Chemother* 2008;62:1281–1284.
25. Seidler MJ, Salvenmoser S, Müller F-M. *Aspergillus fumigatus* forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. *Antimicrob Agents Chemother* 2008;52:4130–4136.
26. Barchiesi F, Calabrese D, Sanglard D, Falconi Di Francesco L, Caselli F, et al. Experimental induction of fluconazole resistance in *Candida tropicalis* ATCC 750. *Antimicrob Agents Chemother* 2000;44:1578–1584.
27. Grenouillet F, Eglin G, Muret P, Devillard N, Leroy J. In vitro induction of voriconazole resistance in *Candida krusei* by azole exposure. In: *Annual ICAAC/IDSA 46th Annual Meeting (Internet)*. Washington, France, 2008. <https://hal.archives-ouvertes.fr/hal-00341443>
28. Snelders E, Camps SMT, Karawajczyk A, Schaftenaar G, Kema GHJ, et al. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS One* 2012;7:e31801.
29. Papadopoulos CJ, Carson CF, Chang BJ, Riley TV. Role of the MexAB-OprM efflux pump of *Pseudomonas aeruginosa* in tolerance to tea tree (*Melaleuca alternifolia*) oil and its monoterpene components terpinen-4-ol, 1,8-cineole, and alpha-terpineol. *Appl Environ Microbiol* 2008;74:1932–1935.
30. Becerril R, Nerín C, Gómez-Lus R. Evaluation of bacterial resistance to essential oils and antibiotics after exposure to oregano and cinnamon essential oils. *Foodborne Pathog Dis* 2012;9:699–705.
31. Piątkowska E, Rusiecka-Ziółkowska J. Influence of essential oils on infectious agents. *Adv Clin Exp Med* 2016;25:989–995.

Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as 'excellent' or 'very good'.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.